The transcriptome of the salivary glands of the female
western black-legged tick *Ixodes pacificus* (Acari: Ixodidae)

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Summary

Sequencing of an *Ixodes pacificus* salivary gland cDNA library yielded 1068 sequences with an average undetermined nucleotide of 1.9% and an average length of 487 base pairs. Assembly of the expressed sequence tags yielded 557 contigs, 138 of which appear to code for secreted peptides or proteins based on translation of a putative signal peptide. Based on the BLASTX similarity of these contigs to 66 matches of *Ixodes scapularis* peptide sequences, only 58% sequence identity was found, indicating a rapid divergence of salivary proteins as observed previously for mosquito and triatomine bug salivary proteins. Here we report 106 mostly full-length sequences that clustered in 16 different families: Basic-tail proteins rich in lysine in the carboxy-terminal, Kunitz-containing proteins (monolaris, ixolaris and penthalaris families), proline-rich peptides, 5-kDa.-, 9.4 kDa.-, and 18.7 kDa.-proteins of unknown functions, in addition to metalloproteases (class PIII-like) similar to reprolysins. We also have found a family of disintegrins, named ixodegrins that display homology to variabilin, a GPIIb/IIIa antagonist from the tick *Dermacentor variabilis*. In addition, we describe peptides (here named ixostatins) that display remarkable similarities to the cysteine-rich domain of ADAMST-4 (aggrecanase). Many molecules were assigned in the lipocalin family (histamine-binding proteins); others appear to be involved in oxidant metabolism, and still others were similar to ixodid proteins such as the anticomplement ISAC. We also identified for the first time a neuropeptide-like protein (nlp-31) with GGY repeats that may have antimicrobial activity. In addition, 16 novel proteins without significant similarities to other tick proteins and 37 housekeeping proteins that may be useful for phylogenetic studies are described. Some of these proteins may be useful for studying vascular biology or the immune system, for vaccine development, or as immunoreagents to detect prior exposure to ticks. Electronic version of the manuscript can be found at http://www.ncbi.nlm.nih.gov/projects/omes/.

Key words: *Ixodes pacificus*, sialome, tick, blood-feeding, Kunitz inhibitor, Lyme disease, vascular biology, vector biology, transcriptome, proteome.

Introduction

Lyme disease is the most prevalent vector-borne disease in the U.S. and is transmitted by the tick vectors *I. scapularis* and *I. pacificus* in eastern and western North America, respectively (Barbour, 1998). Humans usually acquire Lyme disease when an infected nymphal-stage *Ixodes* sp. tick attaches and transmits the spirochete *Borrelia burgdorferi* (Burgdorfer et al., 1985). *I. scapularis* and *I. pacificus* transmit other zoonotic agents besides the Lyme disease spirochete, such as *Anaplasma phagocytophilum* (both species) or *Babesia microti* (*I. scapularis* only) (Barbour, 1998). Transmission is facilitated by tick saliva that operates not only as a carrier for *Borrelia sp*. but also contains a large repertoire of molecules that counteract the host response to injury (Ribeiro and Francischetti, 2003), allowing ticks to feed for days (Sonenshine, 1985). Accordingly, many biologic activities have been described in tick saliva, including molecules that impair platelet aggregation or neutrophil function (Ribeiro et al., 1985) in addition to coagulation inhibitors such as ixolaris and penthalaris that block Factor VIIa/tissue factor complex (Francischetti et al., 2002a; Francischetti et al., 2004a) and SALP 14, which targets Factor Xa (Narasimhan et al., 2002). Enzymes such as a kininase that degrades bradykinin (Ribeiro and Mather, 1998), an apyrase that destroys ADP (Ribeiro et al., 1985), and a metalloprotease with fibrin(ogen)olytic activity (Francischetti et al., 2003) also have been reported. Tick saliva is also rich in small molecules such as prostacyclin, a potent inhibitor of platelet activation and strong inducer of vasodilation (Ribeiro et al., 1988).

As for the immune system, an inhibitor of the alternative complement pathway exists in ixodid tick saliva (Valenzuela et al., 2000). Immunomodulators affecting NK cell function (Kopecky and Kuthejlova, 1998)—in addition to inhibitors of the proliferation of T lymphocytes and an IL-2 binding activity—also are present in this secretion (Ramachandra and Wikel 1992; Gillespie et al., 2001). Finally, saliva is important in transmission of tick-borne pathogens, as it may enhance pathogen transmission (for a review, see Wikel, 1999).

The pace of discovery of tick salivary proteins has been greatly increase by novel molecular biology techniques and bioinformatics analysis (Ribeiro and Francischetti, 2003). Our goal here has been to further study the complexity of *I. pacificus* salivary glands. We report the full-length clone of 87 novel sequences and discuss their potential role in modulating host inflammatory and immune responses.

Materials and methods

Reagents

All water used was of 18 MΩ quality and was produced using a MilliQ apparatus (Millipore, Bedford, MA, USA). Organic compounds were obtained from Sigma (St. Louis, MO, USA) or as stated otherwise.

Ixodes pacificus *ticks*

Salivary gland cDNA library construction and sequencing

Ticks were collected in northern California by dragging low vegetation with a tick-drag. Salivary glands were excised and kept at -80°C until use. The mRNA from two pairs of *I. pacificus* salivary glands was obtained using a Micro-Fast Track mRNA isolation kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit (Clontech, Palo Alto, CA, USA) as described in detail in the supplemental data in Francischetti et al. (2004b). Cycle sequencing reactions using the DTCS labeling kit (Beckman Coulter, Fullerton, CA, USA) were performed as reported (Francischetti et al., 2004b).

cDNA sequence clustering and bioinformatics

Other procedures were as reported in detail in the supplemental data described in Francischetti et al (2004b). The electronic version of the figures and table can be found at http://www.ncbi.nlm.nih.gov/projects/omes/.

Structural bioinformatics and molecular modeling

Molecular model of the histamine-binding protein-like lipocalin gi 51011604 superimposed with the crystal structure of *Rhipicephalus appendicuatus* histamine-binding protein. The 3D-PSSM web server V2.6.0, found at http://www.sbg.bio.ic.ac.uk/ server was used to generate a model of gi 51011604 based on sequence alignment using PSI Blast, secondary structure prediction and search of a fold database of known structures.

Results and Discussion

Ixodes scapularis and *I. pacificus* are the respective vectors for *B. burgdorferi* in the eastern and western U.S. (Fig. 1). After attachment to the host, infected ticks transmit *B. burgdoferi* after 1–2 days of blood-feeding (Barbour, 1998) via saliva, a secretion that contains a cocktail of bioactive molecules (Ribeiro and Francischetti, 2003). Actually, the identification of the transcripts and proteins present in the salivary gland of ticks such as *I. scapularis* (Valenzuela et al., 2002), *Boophilus microplus* (Santos et al., 2004), and *Rhipicephalus appendiculatus* (Nene et al., 2004) have been identified recently. Here we identified secretory genes from the salivary gland of *I. pacificus* constructing a unidirectional PCR-based cDNA library (see Materials and methods). Next, 735 cDNA were randomly sequenced followed by bioinformatics analysis that included: *i)* clustering at high stringency levels, *ii)* BLAST search against the non-redundant and protein motifs databases, and *iii)* submission of the translated

sequences to the Signal P server (see Materials and methods). This initial approach allowed us to obtain a fingerprint of the protein families or "clusters" present in this particular salivary gland. Several sequences were then selected based on novelty or the protein family it assigns for and extension of their corresponding cDNA were performed until the poly A was reached. Among these clusters, 87 novel full-length cDNA coding proteins or peptides were obtained, most of which appear to be secreted in the saliva.

Our results are presented in Table 1, which describes the sequence size, the presence of a putative signal peptide, the molecular weight of the mature peptide, the isoelectric point, and other parameters. Fifteen large protein families of putative secreted proteins were found. Some sequences appeared to code for housekeeping proteins, whereas others without database hits but containing an open-reading frame with or without signal peptide were considered novel or unknown-function proteins. Considering the diverse roles of putative secreted proteins in blood feeding, a brief description for each protein family is presented below.

Group 1: Basic-tail proteins (BTP)

This family of proteins is highly represented in the salivary glands of both *I. pacificus* and *I. scapularis* ticks. Fig. 2A shows the alignments of the BTP of these ticks where a highly conserved signal peptide indicates their common origin from an ancestral gene. Fig. 2A also shows that the pattern of these sequences contain six cysteines (XnCX14CX3CX18CX9CX4CXn) followed by a basic tail with high content of lysines (Lys, K). On the other hand, some proteins from this family, such as gi 22652868 and gi 22164158, display a negatively charged tail composed of six glutamic acid (Glu, E) anionic residues (Fig. 2A). The evolutionary relationships of BTP were inferred by constructing the phylogenetic tree using the NJ algorithm; a cladogram is shown in Fig. 2B. Of interest, these proteins share sequence similarities to exogenous anticoagulants such as SALP 14 (gi 15428308) from *I. scapularis*. SALP 14 is a FXa inhibitor that appears to interact with the catalytic domain of

FXa and with the so-called exosite (Narasimhan, 2002). Exosites—regions far from the catalytic site and known to determine specificity and affinity of blood coagulation factors toward substrates—also are critical for the assembly of the prothrombinase, a multimolecular complex that leads to thrombin generation (Krishnaswamy, 2005). Targeting these domains appears to be an effective strategy evolved by blood-feeding arthropods to effectively impair blood coagulation. In fact, we recently reported that ixolaris, a FX(a) scaffold-dependent inhibitor of Factor VIIa/tissue factor complex, specifically recognizes the FXa heparin-binding exosite (Monteiro et al., 2004).

The fact that BTP and SALP 14 contain a poly-Lys tail adds an additional layer of anticoagulation, as it directs the inhibitor to negatively-charged membranes (e.g., activated platelets) critical for productive blood coagulation complex assembly (Broze, 1995). As a result, the effective concentration of the inhibitor is increased at sites that are predominantly pro-coagulant. Also, we speculate that FXa—which is usually protected from physiologic inhibitors (e.g., TFPI, heparin/ATIII) when the prothrombinase is fully assembled (Mast and Broze, 1996; Rezaie, 2001)—would be more susceptible to these bifunctional molecules. Demonstration that proteins rich in positively charged residues effectively block the coagulation cascade comes from studies performed with a recombinant *Rhodnius prolixus* salivary lipocalin (nitrophorin-7, NP-7). NP-7 contains a cluster of positively charged residues in the N-terminus and specifically binds to anionic phospholipids, preventing thrombin formation by the prothrombinase (Andersen et al., 2004). Finally, a bifunctional fusion protein containing Kunitz and annexin domains was shown recently to inhibit the initiation of blood coagulation (Chen et al., 2005).

Group 2: Similar to Group 1, but without the basic tail

These sequences contain a cysteine pattern identical to Group 1 peptides except that, remarkably, the poly K tail is missing. Many other amino acids also are not conserved. Sequence

alignment between the Group 1 peptides (containing poly K and poly E) and the peptides similar to Group 1 is shown in Fig. 3A. Fig. 3B shows that these proteins come from a common ancestor that appears to have evolved to display different functions. The function of the peptides of Group 2 deserves further investigation.

Group 3: Kunitz-containing proteins

Kunitz domains are about 60 residues and contain 6 specifically spaced cysteines (XnCX8CX15CX7CX12CX3CXn) that form disulfide bonds typically represented by bovine pancreatic trypsin inhibitor (BPTI). In most cases, they are reversible inhibitors of serine proteases that bind the active site (Laskowski and Kato, 1980); however, Kunitz inhibitors such as the dendrotoxins from *Dendroaspis angusticeps* snake venom block K+ channel but display negligible protease inhibitory properties (Harvey, 2001). Kunitz-containing proteins also interact with protease exosites (Monteiro et al., 2004) or platelets (Mans et al., 2002). Of note, sequencing the *I. pacificus* cDNA library yields a number of proteins containing Kunitz-like domains.

The alignment of BPTI, snake venom, and *I. scapularis* and *I. pacificus* single Kunitz-like proteins is shown in Fig. 4A. Some *I. pacificus* proteins contain one-Kunitz-like domain, here named the Monolaris-1 family (or "similar to 6.5- to 8.4-kDa proteins from *I. scapularis*"). These molecules display the following cysteine pattern: XnCX8CX18CX5CX12CX3CXn. Other single-Kunitz sequences present in *I. scapularis* belong to the Monolaris-2 family (or "similar to 7.9- to 8.7-kDa proteins from *I. scapularis*") and display the sequence pattern XnCX8CX15CX8CX11CX3CXn. We could not, however, find members of the Monolaris-2 family sequences in our *I. pacificus* cDNA library. Fig. 4A also shows that the well-known tick anticoagulant peptide from the soft tick *Ornithodoros moubata* (Waxman et al., 1990) has Kunitz-like folding with the sequence pattern XnCX9CX17CX5CX15CX3CXn. At present, the functions of Monolaris-1 and -2 are unknown,

but they may target specific proteases. The philogenetic tree shown in Fig. 4B suggests that snake venom peptides containing Kunitz domains (non-neurotoxic or neurotoxic) and the tick families of Monolaris and basic tail peptides have diverged into two different main groups from a commom ancestor, suggesting that these proteins have evolved to perform different functions.

Additionally, cDNAs were sequenced coding for proteins containing two- or five-Kunitz domains. These proteins share sequence similarity to ixolaris (Francischetti et al., 2002b) and penthalaris (Francischetti et al., 2004a), two *I. scapularis* TFPI salivary proteins that prevent initiation of blood coagulation through specific inhibition of the Factor VIIa/tissue factor complex. It is possible that these proteins block other proteases (Ruf, 2004) or affect angiogenesis (Hembrough et al., 2004).

Fig. 4C depicts the predicted secondary folding of *I. scapularis* and *I. pacificus* Kunitz-like-containing proteins based on the crystal structure determined for BPTI (Huber et al., 1974).

Group 4: Proline-rich proteins

Group 3 cDNA sequences code for short peptides of mature molecular mass ranging from 3.5–4.8 kDa of both basic and acidic nature (Table 1). Alignments and cladograms (presented in Fig. 5A and 5B, respectively), show that all sequences are relatively glycine and proline rich in both *I. pacificus* and *I. scapularis* salivary glands. Some sequences display weak matches to proteins annotated as collagen in the NR database; these possess two conserved cysteine residues in the mature peptide and remarkable conservation of the secretory signal peptide (Fig. 5). Most amino acids of the predicted signal secretory peptide are conserved, versus few on the mature peptide, suggesting functional diversity. The possible function of these peptides remains to be characterized, but taking into account its similarity to collagen, it may somehow affect vascular biology through inhibition of cell-cell, cell-matrix, or cell-ligand interactions. These peptides may also function as adhesive molecules to cement the tick into their host's skin.

Group 5: Similar to I. scapularis *18.7-Kda protein*

This group of proteins (table 1) is similar to orthologs described in *I. scapularis* and code for an acidic putative protein of unknown function. Only low e values have been found when compared with proteins in the NR database including coagulation factor X (gi 9837158, e value 0.069), venom metalloprotease acurhagin precursor (gi 4689408; e value 3.8), and proprotein convertase subtilin (gi 51771463, e value 8.4). Accordingly, this family of proteins may have evolved from a protease precursor; however, any functional assignment will be possible only after testing the recombinant protein in screening assays.

Groups 6 and 7: Similar to I. scapularis *5-kDa protein and 9.4-kDa protein*

Groups 6 and 7 code for basic proteins of ~ 5 kDa and 9.4 kDa that also are present in *I. scapularis*. No protein motif was identified for either protein; accordingly, the function of these proteins is not evident.

Group 8: Metalloprotease

These enzymes are capable of hydrolyzing various components of the extracellular matrix including fibrinogen and fibronectin and reportedly affect endothelial cells, leading to apoptosis. These enzymes are organized into four classes, PI through PIV, according to size and domain composition (Bjarnason and Fox, 1995).

Our library contains a truncated cDNA that codes for a mature metalloprotease similar to one described in *I. scapularis* (gi 31322779) (Francischetti et al., 2003) and *I. ricinus* (gi 5911708). The alignment of the mature metalloproteases from *I. pacificus*, *I. scapularis*, and *I. ricinus*, where the zinc-binding motif HExxHxxGxxH common to these enzymes was

identified, is shown in Fig. 6A. Fig. 6B compares the PIII class of matalloproteases from snake

venom and the *I. pacificus*, *I. scapularis* and *I. ricinus* enzymes. It is clear that enzymes from

both genera have pre-, pro-, metalloprotease, disintegrin-like, and cysteine-rich-like domains;

however, the *Ixodidae* disintegrin-like and cysteine-rich like domains are significantly shorter in

the number of amino acid residues when compared with the corresponding domains of

metalloproteases from the reprolysin family (Bjarnasson and Fox, 1995). We suggest that this

pattern of cysteines confer a different specificity for these enzymes. This family of proteins also

appears to account for the α-fibrinogenase and fibrinolytic activity recently reported for

I. scapularis saliva (Francischetti et al., 2003). Degradation of fibrinogen and fibrin are

associated with inhibition of platelet aggregation and clot formation. Metalloproteases also may

interact with endothelial cell integrins, leading to apoptosis and inhibition of angiogenesis

(Ribeiro and Francischetti, 2003).

Group 9: GPIIb/IIIa antagonists from the short neurotoxin family

Inhibitors of platelet aggregation that targets the fibrinogen receptor (GPIIbIIIa, integrin αIIbβ3)

has been described in the hard tick *Dermacentor variabilis* (variabilin) and the soft ticks,

Ornithodoros moubata (disagregin) and *O. savignyi* (savignygrin) (Karczewski et al. 1994;

Wang et al. 1996; Mans et al. 2002). Savignygrin belongs to the Kunitz-BPTI family and

presents the integrin RGD-recognition motif on the substrate binding loop of the Kunitz fold

(Mans et al. 2002). In contrast, variabilin, in contrast possesses an RGD-motif in its C-terminal

region that is not flanked by cysteines (Wang et al. 1996). A search for possible GPIIb/IIIa

antagonists with RGD-motifs and flanking cysteines, termed the Ixodegrins, identified one

candidate in *I. pacificus* and several homologs in *I. scapularis* (Table 1). It is clear that the

Ixodegrins are related to variabilin, but do possess flanking cysteines. Variabilin probably

possesses a flanking disulphide motif too, but was missed due to the technical difficulties in

identifying cysteines correctly during N-terminal sequencing. Database searches using SAM-T99

(Karplus et al. 1998), a program that utilizes hidden Markov models to find remote homologous

sequences, identified dendroaspin as the highest hit. Dendroaspin, also known as mambin, is part

of the short neurotoxin family found in elapid snakes (McDowell et al. 1992; Williams et al.

1992; Sutcliffe et al. 1994). Strikingly, the RGD-active site loop (loop3) is conserved between

snake and tick integrin antagonists (Fig. 7A). This includes the flanking cysteines involved in a

disulphide bond that constricts the RGD-loop conformation and the flanking prolines that was

shown to be important for presentation of the RGD sequence (Lu et al. 2001). The tick inhibitors

maintain loops 2 and 3 of the short neurotoxin fold, but do not possess the N-terminal loop 1 and

the C-terminal extension (Fig. 7A). This makes them the shortest members of the short

neurotoxin family described to date, with only 39 amino acids forming the core active fold.

Phylogenetic analysis of the neurotoxin family indicates that dendroaspin and tick inhibitors

group within one clade to the exclusion of the other short neurotoxins (Fig. 7B). This suggests

either an extreme form of convergent evolution, where ticks and elapid snakes used the same

protein fold to evolve the same function or raises the possibility that ticks or snakes acquired the

ancestral protein via a horizontal gene transfer event or that there is a true evolutionary

relationship between the ixodegrins and short neurotoxins. The fact that orthologs of the

Ixodegrins are present in both *Ixodes* (prostriate) and *Dermacentor* (metastriate) ticks, suggests

that this inhibitor was present in the last common ancestor of hard ticks. Snakes evolved most of

their venom properties approximately 60-80 million years ago (Fry, 2005), whereas most hard

tick genera diverged at least 110 million years ago or earlier (Klompen et al. 1996). If tick and

snake proteins are related, then the ancestral gene may have a platelet antagonist function and the

neurotoxic properties (and the rest of the short neurotoxin fold - loop1 and the C-terminal extension) evolved later. In contrast, soft ticks in the genus *Ornithodoros* evolved integrin antagonists from the BPTI-fold which suggests that hard and soft ticks evolved different strategies to obtain a blood meal (Mans et al. 2002b; Mans and Neitz, 2004). Accordingly, ixodegrin may affect platelet or neutrophil integrin function or neutrophil function.

Group 10: Ixostatin family, or short-coding cysteine-rich peptides ("thrombospondin").

The two sequences in Group 11 match a sequence deposited in the NR database from *I. scapularis*; alignments are shown in Fig. 8A. These sequences have been annotated "thrombospondin" (gi 15428290), but thrombospondin motifs are lacking. On the contrary, these short coding region cysteine-rich peptides—here named ixostatins—are remarkably similar to the cysteine-rich domain of ADAMTS (Fig.8B). Of note, ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs), also known as aggrecanase, are enzymes involved in cartilage cleavage (Flannery et al., 2002). The role of the cysteine-rich domain of ADAMTS proteases is unknown, but it is postulated to interact with integrins and/or other attachment motifs of cells and matrix proteins (Porter et al., 2005). Accordingly, the ixostatin family of peptides could be involved in disruption of platelet aggregation or neutrophil function, cell-matrix interactions, or inhibition of angiogenesis (Porter et al., 2005). The protein modules of ixostatin and of ADAMST-4 are compared in Fig 8C.

Group 11: Histamine-binding proteins (lipocalins)

Group 11 contains sequences with similarities to histamine-binding proteins discovered in the saliva of *Rhipicephalus appendiculatus* ticks (Paesen et al., 1999). The alignments of these

sequences (Fig. 9A) reveal that they do not display a highly conserved signal peptide which suggest that they may not share a common ancestor. In addition, the mature proteins contain few consensus sequences indicating that they may have diverged to perform distinct functions (Fig. 9B). This contention is also supported by the cladogram presented in Fig. 9B. It is likely that these proteins function by binding small ligands such as histamine, serotonine, and adrenaline (Andersen et al., 2005). Fig. 9C shows a predicted 3-D model for sequence gi 51011604 that has an e value of -768 for HBP from *R. appendiculatus*. The figure shows amino acid side chains of the histamine-binding protein from *R. appendiculatus* (red) surrounding the bound histamine ligand with the corresponding residues for gi 51011604 shown in cyan. In the histamine-binding protein, the imidazole ring of the ligand is stabilized by surrounding aromatic residues, while in the *I. scapularis* protein the binding pocket remains hydrophobic, fewer aromatic residues are present, suggesting a different ligand specificity. Polar residues (Tyr 36 and Glu 135) forming electrostatic interactions with the aliphatic amino group of histamine in the histamine-binding protein are conserved in gi 51011604 suggesting the possibility of a similar role in this protein.

Group 12: Neuropeptide-like (npl-31) protein with GGY repeat

A cDNA coding for a protein that shows remarkable sequence homology to a neuropeptide-like protein (npl-21) described in *Caenorhabditis elegans* (Nathoo et al., 2001). This family of peptides displays a potent antimicrobial activity toward *Drosophila coniospora*, *Neurospora crassa*, and *Aspergillus fumigatus* (Couillault et al., 2004). Identification of these peptides in ticks reinforces the notion that saliva contains a cocktail of antimicrobial peptides. These peptides may prevent growth of yeast and bacteria that, *per se,* can elicit an inflammatory/immune response that may be detrimental to the feeding behavior of the attached

ticks. Expression of these molecules is particularly important *vis-à-vis* the remarkably immunosupressive property of the saliva (Wikel, 1999) that helps ticks to feed for days but otherwise creates an appropriate environment for pathogen overgrowth. The sequence alignments for *C. elegans* npl-21 and *I. pacificus* npl-21-like proteins are presented in Fig. 10A and the cladogram i in Fig. 10B. This is the first time that this family of antimicrobial peptides has been identified in the salivary gland of a blood-sucking arthropod.

Group 13: Oxidant metabolism

Proteins with similarity to glutathione peroxidase and a putative secreted superoxide dismutase were found (Table 1). These sequences categorize the prominent salivary gland proteins in *I. pacificus* and demonstrate the presence of a potent antioxidant in tick saliva. Of interest, cluster F12_IPL_P23 has sequence similarity to SALP 25, a protein that catalyzes the reduction of hydrogen peroxide in the presence of reduced glutathione and glutathione reductase (Das et al., 2001). The functions of these proteins are likely related to maintenance of the physiologic redox of cellular intracellular milieu or to modulation of the extracellular levels of pro-oxidants often associated with inflammatory events.

Group 14: Similar to other ixodid proteins

A number of sequences show sequence homology to proteins from *ixodid* described before. We have found sequences similar to SALP 15, a immunodominat protein in *I. scapularis* (Das et al. 2001), and to ISAC, the anti-complement from *I. scapularis* (Valenzuela et al., 2002). We also have found sequences similar to domain 8 of human ADAMS and Factor VII.

Group 15: Novel, unknown

Some sequences containing a signal peptide and a stop codon and with a clear open reading frame were without database hits and were characterized as unknown-function proteins.

Assignment of function for these proteins will only be possible after expressing and screening for testable biologic activities.

Group 16: Housekeeping cDNA

Thirty-seven sequences with homology to housekeeping protein are given in Table 1. They assign to ribosomal, glutathione S-transferase, vacuolar aasorting proteins, cytochrome, ATP synthase subunit, and NADH-ubiquinone oxidoreductase, among other molecules. In addition, housekeeping proteins may be useful in phylogenetic studies (Black and Piesman, 1994).

I. pacificus *salivary gland protein diversity:*
modulators of vascular biology and candidates for an anti-saliva experimental vaccine

We describe the set of cDNA present in the salivary glands of *I. pacificus* salivary gland. Our library contains a remarkably large degree of redundancy, as shown by the many related mRNAs. It appears that the long evolutionary history of ticks may be responsible for the complexity of transcripts reported here. Also, many protein families we have identified were found previously in *I. scapularis* salivary glands (Valenzuela et al., 2002) which confirms the diverse nature of these secretions compared with the salivary composition of fast feeders such as sand flies (Charlab et al., 1999) and mosquitoes (Francischetti et al., 2002a). This variability in the tick salivary gland is consistent with the high polymorphism of salivary proteins among individual ticks analyzed by SDS-PAGE (Wang et al., 1999). The adaptive role of this diversity appears to be explained at least in part by a gene-duplication phenomenon. This contention is supported by the diversity of sequences containing Kunitz-like domains in addition to a weak similarity observed among members of the lipocalin family of proteins reported here. It may be that these inhibitors have evolved to inhibit different proteases or to bind to different ligands (Andersen et al., 2005). It is also plausible that gene duplication may help ixodid ticks to evade

the immune system. If so, this may help to explain why hard ticks can remain attached to many hosts for days without apparent detrimental effects (Ribeiro and Francischetti, 2003).

The functions of many tick sequences described in this paper are unknown. Cloning and expressing select cDNA will help in the identification of molecule specificity and, accordingly, our understanding of how ticks successfully feed on blood. It also may provide tools to understand vascular biology and the immune system. A diagram with the putative targets of salivary proteins and how they may affect vascular biology is shown in <u>Fig. 11</u>. Finally, defining the most abundant antigens or those that may effectively help ticks to feed or transmit *Borrelia* could be an effective approach to develop a protective vaccine directed toward tick salivary molecules (Lane et al., 1999).

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Figure Legends

Fig. 1. Established and reported distribution of the Lyme disease vectors *Ixodes scapularis* (*I. dammini*) and *Ixodes pacificus* by county, United States, 1907−1996. Distribution was reported by the Centers for Disease Control and Prevention and can be found at http://www.cdc.gov/ncidod/dvbid/lyme/tickmap.htm.

Fig. 2. Group 1: Basic tail proteins (BTP). (A) Alignments of peptides from *I. pacificus* (Table 1) and *I. scapularis* BTP deduced from cDNA libraries. Conserved amino acid residues are shown in black background. Lysine residues (K) are shown in bold (Poly K, lysine tail). (B) The bar represents the degree of divergence among sequences.

Fig. 3. Group 2: Similar to Group 1, without basic tail. (A) Alignment of Group 2 peptides (Table 1). Conserved amino acid residues are shown in gray background. (B) The unrooted cladogram of all sequences. The bar represents the degree of divergence among sequences.

Fig. 4. Group 3: Kunitz-containing proteins. (A) Alignment of Group 3 peptides (Table 1) with single Kunitz-containing protein from snake venoms. Conserved amino acid residues are shown in gray background. (B) The phylogram was constructed using protein from snake venom single-kunitz (neurotoxic or non-neurotoxic from Elapidae and Viperidae families) and tick salivary gland, plus BPTI (all accession numbers are depicted). The bar represents the degree of divergence among sequences. (C) Predicted secondary folding of Kunitz containing proteins from Ixodidae sp. based on BPTI folding (Huber et al., 1974).

Fig. 5. Group 4: Proline-rich peptides. (A) Alignment of Group 4 peptides (Table 1). Signal peptide is shown in gray background, and conserved amino acid residues are shown in black background. (B) The unrooted cladogram of all sequences. The bar represents the degree of divergence among sequences.

Fig. 6. Group 8: Metalloproteases. (A) Alignment of metalloproteases from *I. pacificus* (Ip) (Table 1), *I. scapularis* (Is), and *I. ricinus* (Ir). The characters in bold represent the conserved Zn binding motif present in the catalytic domain. Asterisks, colons, and stops below the sequences indicate identity, high conservation, and conservation of the amino acids, respectively. (B) Diagram comparing the protein motifs (pre, pro, catalytic, disintegrin-like, and cysteine rich-like domains) of class III metalloproteases from snake venoms and tick class III-like metalloproteases.

Fig. 7: Group 9: Ixodegrin: disintegrins. (A) Alignment of the ixodegrins from *Ixodes pacificus* (Ixodegrin_Ip), *I. scapularis* (Ixodegrin_Sc1/2/3), variabilin and dendroaspin. Shadowed in gray are conserved cysteine regions and the RGD motif. Also indicated are the loops and disulphide bond pattern of the short neurotoxin fold and the inferred disulphide bond patterns of the ixodegrins. (B) A neighbor-joining tree of the short neurotoxin family. Indicated are different functional clades found for the family. Snake proteins are referred to by their Swiss-Prot name. Black circles indicate confidence levels >70% from 10 000 bootstraps.

Fig. 8. Group 10: Ixostatin: short coding cysteine-rich peptides. (A) Alignment of Group 9 peptides from *I. pacificus* (Table 1) and *I. scapularis*. Conserved amino acid residues are shown in black background. (B) Alignment between ixostatin and the cysteine rich-domain of ADAMST-4 (aggrecanase). (C) Diagram comparing the protein motifs (pre, pro, catalytic, disintegrin-like, cysteine rich-like, and spacer domains) of ADAMST-4 (Flannery et al., 2002) and ixostatin.

Fig. 9. Group 11: Histamine-binding proteins (lipocalins). (A) Alignment of Group 10 peptides from *I. pacificus* (Table 1). Conserved amino acid residues are shown in black background. (B) The unrooted cladogram of all sequences. The bar represents the degree of divergence among sequences. (C) The figure shows amino acid side chains of the histamine-binding protein from *R. appendicuatus* (red) surrounding the bound histamine ligand. The corresponding residues for gi 51011604 are shown in cyan. In the histamine-binding protein, the imidazole ring of the ligand is stabilized by surrounding aromatic residues. In the *I. scapularis* protein the binding pocket remains hydrophobic, fewer aromatic residues are present, suggesting a different ligand specificity.

Fig. 10. Group 12: Neuropeptide-like (npl-31) peptides. (A) Alignment of Group 11 peptides from *I. pacificus* (Table 1) and *I. scapularis*. Conserved amino acid residues are shown in gray background. (B) The unrooted cladogram of all sequences. The bar represents the degree of divergence among sequences.

Fig. 11. Negative modulators of vascular biology are present in *I. pacificus and I. scaularis* saliva. Vascular injury is accompanied by vasoconstriction and activation of the extrinsic and intrinsic pathways of blood coagulation (Broze et al., 1995). Vasoconstriction is mediated by molecules such as serotonine that may be removed by salivary protein with a lipocalin folding (Andersen et al., 2005). The extrinsic pathway is initiated by tissue factor/factor VIIa complex and effectively blocked by ixolaris (Francischetti et al., 2002b) and penthalaris (Francischetti et al., 2004a). FXa generated by the intrinsic or extrinsic Xnase may be inhibited by Group 1 peptides containing a basic tail that may prevent productive prothrombinase complex assemble (Rezaie, 2000; Narasimhan et al., 2002; Andersen et al., 2004, Monteiro et al., 2004). Platelet, neutrophil, and endothelial cell function may be affected by Ixodegrins (disintegrins) or Ixostatins (short-coding cysteine-rich peptides). Metalloproteases appear to cleave fibrinogen and fibrin, therefore inhibiting platelet aggregation and clot formation (Francischetti et al., 2003). Metalloproteases also may affect endothelial cell function and angiogenesis (Ribeiro and Francischetti, 2003). The intrinsic pathway that is activated by contact (Broze et al., 1995) leads to bradykinin formation, a peptide that increases vascular permeability and induces pain. Bradykinin is degraded by a salivary kininase, thus preventing its pro-inflammatory effects (Ribeiro and Francischetti, 2003).